Title

Combined therapy comprising nemorubicin and a Cyclooxygenase-2 inhibitor

Field of the invention

The present invention pertains to the field of neoplastic diseases therapy. In particular, this invention relates to a method for treating cancer in a subject in need of such a treatment, said method comprising administering to the patient a therapeutically effective amount of a morpholinyl anthracycline derivative a pharmaceutically acceptable salt or a metabolite thereof and a ciclooxygenase-2 (Cox-2) selective inhibitor. The invention also relates to compositions or packaged units comprising a morpholinyl anthracycline derivative and a Cox-2 inhibitor.

Background of the invention

Cancers are a leading cause of death in humans and animals; surgery, radiation and chemotherapy are the useful means to fight cancers.

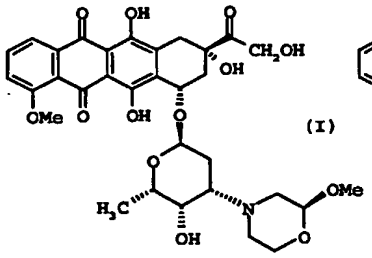
In particular, combined chemotherapy, designed to treat cancer by using more than one drug in combination or association, is a well-accepted modality of treatment of neoplastic diseases such as cancer. Several efforts have been and are still being undertaken in order to select antitumor combinations more and more active and safe to be administered to a patient suffering from a cancer.

The increase of the antitumor efficacy of a known antitumor compound by administering the same in combination with one or more different antitumor compounds in order to reduce the toxic effects of the individual agents when used alone, and in some instances because the combination has greater efficacy than when either agent is used alone, is a strongly felt need in the field of anticancer therapy.

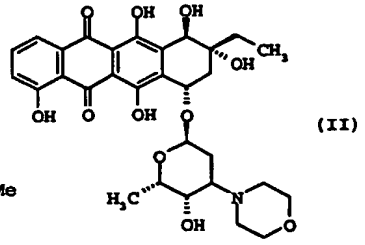
The present invention fulfills such a need by providing a morpholinyl anthracycline derivative a pharmaceutically acceptable salt or a metabolite thereof administered in combination with a Cox-2 inhibitor. Among the several advantages found to be achieved by the present invention, therefore, may be noted the provision of an effective method for the treatment of cancer, the provision of such methods that provided beneficial properties that are comparable to or superior to those provided by known and conventional methods of treatment for these conditions, and the provision of compositions, pharmaceutical compositions and kits to effect these methods.

Description of the invention

It is therefore an object of the present invention combined preparations, comprising a morpholinyl anthracycline derivative having formula (I), formula (II)



(I)



(II)

a pharmaceutically acceptable salt or a pharmaceutically active metabolite thereof, administered in combination with a Cox-2 inhibitor.

According to the present invention, a preferred morpholinyl anthracycline derivative is the morpholinyl anthracycline derivative of formula (I), more particularly in the form of its hydrochloride salt.

As used herein, the term "metabolite" embraces all derivatives resulted from an enzymatic biotransformation of a morpholinyl anthracycline derivative according to the invention. The chemical reactions of enzymatic biotransformation are classified as phase-I or phase-II reactions.

As used herein, the term "pharmaceutically acceptable salt" refers to those salts, which retains the biological effectiveness and properties of the parent compound. Such salts include acid addition salt which is obtained by reaction of the free base of the parent compound with inorganic acids such as hydrochloric acid, hydrobromic acid, nitric acid, phosphoric acid, sulfuric acid and perchloric acid and the like, or with organic acids such as acetic acid, maleic acid, metthanesulfonic acid, ethanesulfonic acid, tartaric acid, citric acid, succinic acid and the like, preferably hydrochloric acid.

The morpholinyl anthracycline of formula (I) namely 3'desamino-3'[2(S) methoxy-4-morpholinyl] doxorubicin, also known as nemorubicin, is a doxorubicin (DX) derivative different from classical anthracyclines, obtained with the substitution of the $-NH_2$ at position 3' in the sugar moiety with a methoxymorpholinyl group.

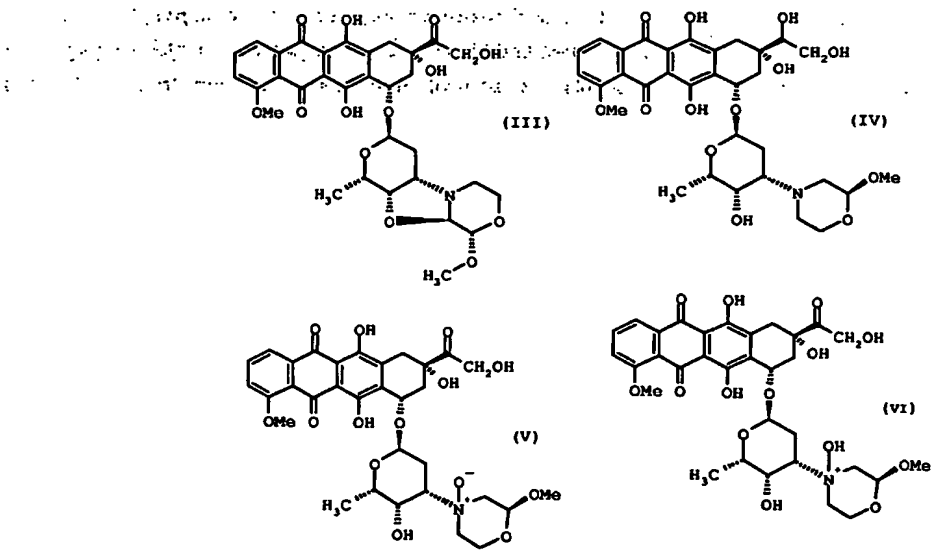
As used herein, the term "nemorubicin" includes, unless otherwise specified, the morpholinyl anthracycline derivative of formula (I) and its pharmaceutically acceptable salts, especially the hydrochloride salt.

Nemorubicin, synthesized in the course of a research program aimed at identifying new anthracyclines with novel modes of action, effective against anthracycline resistant tumors and possessing broad spectrum of antitumor activity, was disclosed and claimed in Bargiotti et al., US patent No. 4,672,057.

Compared to DX, nemorubicin is significantly more potent *in vivo* than *in vitro*. This observation suggested an *in vivo* metabolism of the drug to potent metabolite/s. In *in vitro* experiments, in the presence of mouse,

rat and human liver microsomal enzymes, nemorubicin is metabolized to potent metabolite/s. Microsomal activation appears to occur also *in vivo* since nemorubicin is highly effective on liver metastases.
Nemorubicin is currently undergoing clinical evaluation; clinical data obtained so far suggest an interesting affinity of nemorubicin for liver lesions, even in tumor types resistant to conventional chemotherapy.

Examples of identified metabolites of nemorubicin are compounds of the below formulae (III) to (VI)




The metabolites of the above formulae (III), (IV) and (V) have been described, e.g., in Beutz-Riche et al, Fundamental & Clinical Pharmacology 15 (2001), 373-378.
Fraier et al. have developed and validated a selective and sensitive liquid chromatography-tandem mass spectrometry (LC-MS-MS) method for quantitative determination of nemorubicin, and its reduced

metabolite of the above formula (IV) in human plasma (see J. of Pharmaceutical and Biomedical Analysis 2002, 30(3), 377-389).

The metabolites of the above formulae (III), (IV) (V) and (VI) are active antitumor compounds "per se".

The preparation of the compound of formula (III) may be carried out, for example, following the procedure disclosed in GB 2325067.

The preparation of the compound of formula (IV) may be carried out, for example, following the procedure disclosed in GB 2247885.

The preparation of the compounds of formula (V) and (VI) may be carried out, for example, following the procedure disclosed in GB 2294495.

The compounds of formulae (II) to (VI) may also exist in the form of a pharmaceutically acceptable salt, in this case preferred salts are hydrochloride salts.

In a further aspect, the present invention embraces a combined preparation comprising a compound of the above formula (III), (IV) (V) or (VI) administered in combination with a Cox-2 inhibitor.

MX2, a morpholinyl anthracycline belonging to the family of 3'-deamine-3'(4-morpholinyl) derivatives of 10-hydroxy-13-deoxocarminomycin, was described and claimed in Otake et al., US patent no. 4,710,564.

MX2 is active *in vitro* and *in vivo* on tumor cells resistant to anthracyclines and presenting the multi-drug resistant phenotype.

No cross-resistance was observed on tumor cells resistant to CTX, L-PAM and cDDP.

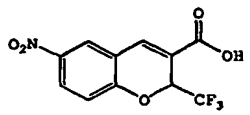
MX2 is active *in vivo* after i.p., i.v. and oral administration, with good antileukemic and antitumor activity on murine and human tumor models. MX2 is highly lipophilic and less cardiotoxic than DX. The major dose limiting factor of MX2 is myelosuppression.

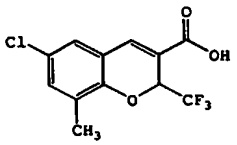
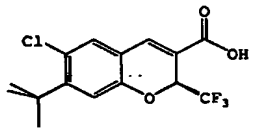
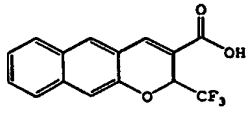
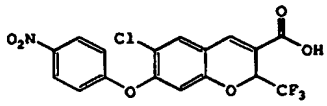
Inducible cyclooxygenase-2 (Cox-2) is an immediate-early response gene. Extensive studies have recognized its overexpression in several carcinomas and its implication in carcinogenesis and tumor progression. Recent clinical studies have indicated that the presence of Cox-2 in human lung and colon is associated with poor prognosis and that overexpression of Cox-2 might be one of the leading factors in hepatic carcinogenesis (Clin. Cancer Res. 5:1001-5, 1999; Clin. Cancer Res. 6:4064-6, 2000; Clin. Cancer Res. 7:1325-32, 2001).

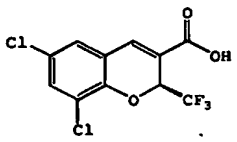
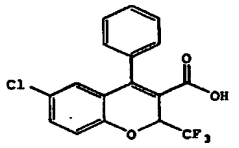
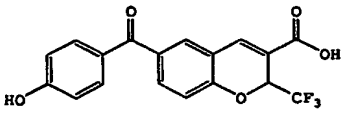
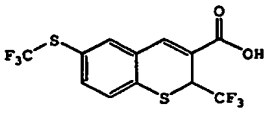
The term "cyclooxygenase-2 inhibitor", as used herein, embraces compounds, which selectively inhibit Cox-2 over cyclooxygenase-1, and also includes pharmaceutically acceptable salts of those compounds.

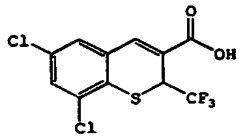
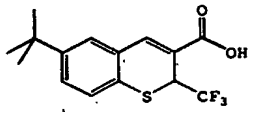
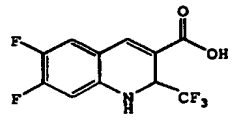
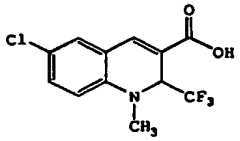
Examples of Cox-2 selective inhibitors to be used in combination with a morpholinyl anthracycline derivative according to the invention are chromene Cox-2 selective inhibitors listed in Table 1.

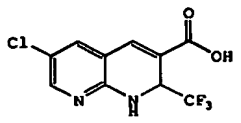
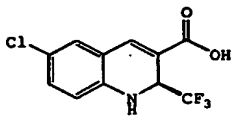
Table 1. Examples of Chromene Cox-2 Selective Inhibitors

| Compound Number | Structural Formula |
|---|---|
| B-3 |  6-Nitro-2-trifluoromethyl-2H-1 -benzopyran-3-carboxylic acid |

| Compound Number | Structural Formula |
|---|---|
| B-4 |  6-Chloro-8-methyl-2-trifluoromethyl -2H-1-benzopyran-3-carboxylic acid |
| B-5 |  ((S)-6-Chloro-7-(1,1-dimethylethyl)-2-(trifluo romethyl-2H-1-benzopyran-3-carboxylic acid |
| B-6 |  2-Trifluoromethyl-2H-naphtho[2,3-b] pyran-3-carboxylic acid |
| B-7 |  6-Chloro-7-(4-nitrophenoxy)-2-(trifluoromethyl)-2H-1- benzopyran-3-carboxylic acid |

| Compound Number | Structural Formula |
|---|---|
| B-8<br>(SD-8381) |  <br>((S)-6,8-Dichloro-2-(trifluoromethyl)-2H-1-benzopyran-3-carboxylic acid |
| B-9 |  <br>6-Chloro-2-(trifluoromethyl)-4-phenyl-2H-1-benzopyran-3-carboxylic acid |
| B-10 |  <br>6-(4-Hydroxybenzoyl)-2-(trifluoromethyl)-2H-1-benzopyran-3-carboxylic acid |
| B-11 |  <br>2-(Trifluoromethyl)-6-[(trifluoromethyl)thio]-2H-1-benzothiopyran-3-carboxylic acid |

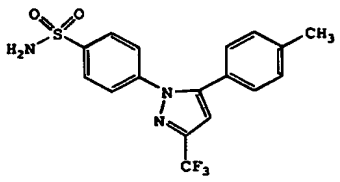
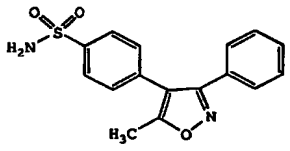
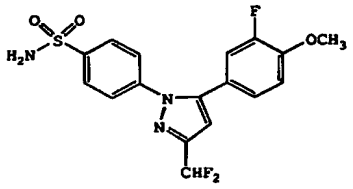
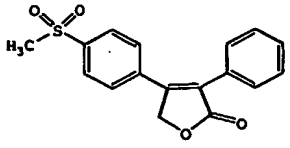
| Compound Number | Structural Formula |
|---|---|
| B-12 |  6,8-Dichloro-2-trifluoromethyl-2H-1-benzothiopyran-3-carboxylic acid |
| B-13 |  6-(1,1-Dimethylethyl)-2-(trifluoromethyl)-2H-1-benzothiopyran-3-carboxylic acid |
| B-14 |  6,7-Difluoro-1,2-dihydro-2-(trifluoro methyl)-3-quinolinecarboxylic acid |
| B-15 |  6-Chloro-1,2-dihydro-1-methyl-2-(trifluoro methyl)-3-quinolinecarboxylic acid |

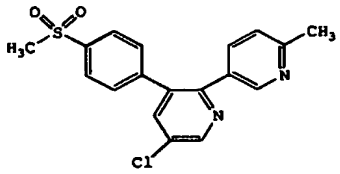
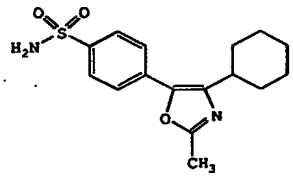
| Compound Number | Structural Formula |
|---|---|
| B-16 |  6-Chloro-2-(trifluoromethyl)-1,2-dihydro [1,8]naphthyridine-3-carboxylic acid |
| B-17 |  ((S)-6-Chloro-1,2-dihydro-2-(trifluoro methyl)-3-quinolinecarboxylic acid |

The compound SD-8381, shown as the structure in figure B-8 above, is a preferred chromene-type Cox-2 selective inhibitor to be used in combination with a morpholinyl anthracycline derivative according to the present invention. The sodium salt form of the compound is preferred. Further information about SD-8381 can be found in U.S. Patent No. 6,034,256.

In a further preferred embodiment the cyclooxygenase-2 inhibitor to be used in combination with a morpholinyl anthracycline derivative of the present invention can be selected from the class of tricyclic Cox-2 selective inhibitors listed in Table 2.

Table 2. Examples of tricyclic Cox-2 selective inhibitors

| Compound Number | Structural Formula |
|---|---|
| B-18 (Celecoxib) |  |
| B-19 (Valdecoxib) |  |
| B-20 (Deracoxib) |  |
| B-21 (Rofecoxib) |  |

| Compound Number | Structural Formula |
|---|---|
| B-22 (Etoricoxib) |  |
| B-23 (JTE-522) |  |

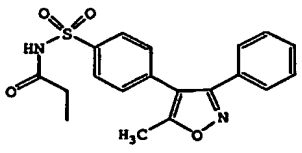
In a still more preferred embodiment the Cox-2 selective inhibitor to be combined with a morpholinyl anthracycline derivative of the present invention can be selected from the group of compounds, illustrated in Table 2, consisting of celecoxib (B-18), valdecoxib (B-19), deracoxib (B-20), rofecoxib (B-21), etoricoxib (MK-663; B-22), JTE-522 (B-23), BMS-347070, or a prodrug thereof.

In an even more preferred embodiment of the invention, the Cox-2 selective inhibitor is selected from the group consisting of celecoxib, rofecoxib and etoricoxib.

In another preferred embodiment of the invention, parecoxib (See, e.g. U.S. Patent No. 5,932,598), having the structure shown in B-24, which is a therapeutically effective prodrug of the tricyclic Cox-2 selective inhibitor valdecoxib, B-19, (See, e.g., U.S. Patent No. 5,633,272), may be

advantageously employed as a source of a cyclooxygenase inhibitor. A preferred form of parecoxib is sodium parecoxib.

Additional information about selected examples of the Cox-2 selective inhibitors discussed above can be found as follows: celecoxib (CAS RN 169590-42-5, C-2779, SC-58653, and in U.S. Patent No. 5,466,823); deracoxib (CAS RN 169590-41-4); rofecoxib (CAS RN 162011-90-7); compound B-24 (U.S. Patent No. 5,840,924); compound B-26 (WO 00/25779); and etoricoxib (CAS RN 202409-33-4, MK-663, SC-86218, and in WO 98/03484).



B-24

The term "pharmaceutically effective amount" shall mean that amount of a drug or pharmaceutical agent that will elicit the biological or medical response of a tissue, system, animal or human that is being sought by a researcher or clinician. This amount can be a therapeutically effective amount.

The term "therapeutically-effective" is intended to qualify the amount of each agent for use in the combination therapy, which will achieve the goal of improvement in disease severity and the frequency of incidence over treatment of each agent by itself, and/or of amelioration of adverse side effects typically associated with alternative therapies.

The combined preparations according to the present invention would be useful for the treatment of cancer. Preferably, the subject methods and compositions of the present invention may be used for the treatment of

neoplasia disorders including benign, metastatic and malignant neoplasias, and also including acral lentiginous melanoma, actinic keratoses, adenocarcinoma, adenoid cystic carcinoma, adenomas, adenosarcoma, adenosquamous carcinoma, astrocytic tumors, bartholin gland carcinoma, basal cell carcinoma, bronchial gland carcinomas, capillary, carcinoids, carcinoma, carcinosarcoma, cavernous, cholangiocarcinoma, chondosarcoma, choriod plexus papilloma/ carcinoma, clear cell carcinoma, cystadenoma, endodermal sinus tumor, endometrial hyperplasia, endometrial stromal sarcoma, endometrioid adenocarcinoma, ependymal, epitheloid, Ewing's sarcoma, fibrolamellar, focal nodular hyperplasia, gastrinoma, germ cell tumors, glioblastoma, glucagonoma, hemangiblastomas, hemangioendothelioma, hemangiomas, hepatic adenoma, hepatic adenomatosis, hepatocellular carcinoma, insulinoma, intaepithelial neoplasia, interepithelial squamous cell neoplasia, invasive squamous cell carcinoma, large cell carcinoma, leiomyosarcoma, lentigo maligna melanomas, malignant melanoma, malignant mesothelial tumors, medulloblastoma, medulloepithelioma, melanoma, meningeal, mesothelial, metastatic carcinoma, mucoepidermoid carcinoma, neuroblastoma, neuroepithelial adenocarcinoma nodular melanoma, oat cell carcinoma, oligodendroglial, osteosarcoma, pancreatic polypeptide, papillary serous adenocarcinoma, pineal cell, pituitary tumors, plasmacytoma, pseudosarcoma, pulmonary blastoma, renal cell carcinoma, retinoblastoma, rhabdomyosarcoma, sarcoma, serous carcinoma, small cell carcinoma, soft tissue carcinomas, somatostatin-secreting tumor, squamous carcinoma, squamous cell carcinoma, submesothelial, superficial spreading melanoma, undifferentiated carcinoma, uveal melanoma, verrucous carcinoma, vipoma, well differentiated carcinoma, and Wilm's tumor.

The terms "treating" or "to treat" mean to alleviate symptoms, eliminate the causation either on a temporary or permanent basis, or to prevent or slow the appearance of symptoms. The term "treatment" includes alleviation, elimination of causation of or prevention of cancer. Besides being useful for

human treatment, these combinations are also useful for treatment of mammals, including horses, dogs, cats, rats, mice, sheep, pigs, etc.

The term "subject" for purposes of treatment includes any human or animal subject who is in need of the prevention of, or who has cancer, cardiovascular disease, or pain, inflammation and/or any one of the known inflammation-associated disorders. The subject is typically a mammal. "Mammal", as that term is used herein, refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cattle, etc., Preferably, the mammal is a human.

The subject pharmaceutical compositions may be administered to a patient in any acceptable manner that is medically acceptable including orally, parenterally or with locoregional therapeutic approaches such as e.g. implants. Parenteral administration includes administering the constituents of the combined preparation by subcutaneous, intramuscular, intradermal, intramammary, intravenous injections and other administrative methods known in the art. Implants include intra arterial implants, for example, an intrahepatic artery implant.

The administration of the constituents of the combined preparations of the present invention can be made simultaneously, separately or sequentially in any order. Namely, the present invention intends to embrace administration of a morpholinyl anthracycline derivative and a Cox-2 inhibitor in a sequential manner in a regimen that will provide beneficial effects of the drug combination, and intends as well to embrace co-administration of these agents in a substantially simultaneous manner, such as in a single dosage device having a fixed ratio of these active agents or in multiple, separate dosage devices for each agent, where the separate dosage devices can be taken together contemporaneously, or taken within a period of time sufficient

to receive a beneficial effect from both of the constituent agents of the combination.

It is therefore another object of the present invention the use of a morpholinyl anthracycline derivative of formula (I), formula (II), a pharmaceutically acceptable salt or a metabolite thereof for the preparation of a medicament in association with a Cox-2 inhibitor for simultaneous, separate or sequential use for the treatment of cancer.

As an example, the combined therapy of the present invention enhances the antitumoral effects of a morpholinyl anthracycline derivative and of the Cox-2 inhibitor and thus yields a more effective and less toxic treatment for tumors.

The constituents of the combined preparations according to the invention can be administered to a patient in any acceptable manner that is medically acceptable including orally, parenterally, or with locoregional therapeutic approaches such as, e.g., implants. Oral administration includes administering the constituents of the combined preparation in a suitable oral form such as, e.g., tablets, capsules, lozenges, suspensions, solutions, emulsions, powders, syrups and the like. Parenteral administration includes administering the constituents of the combined preparation by subcutaneous, intravenous or intramuscular injections. Implants include intraarterial implants, for example an intrahepatic artery implant.
Injections and implants are preferred administration routes for nemorubicin because they permit precise control of the timing and dosage levels used for administration.
For example, administration of nemorubicin to a patient with a liver cancer may be performed via the hepatic artery.
In a particular embodiment of the present invention, nemorubicin may be administered via the hepatic artery as an infusion; the appropriate dose of nemorubicin, preferably previously dissolved in saline solution, may be mixed with a suitable amount, for example an amount ranging from 1 ml to 100 ml

of an agent, for example iodized oil (LIPIODOL ™), which remains selectively in a liver tumor after its injection through the hepatic artery.

The actual preferred method and order of administration of the constituents of the combined preparations of the invention may vary according to, inter alia, the particular pharmaceutical formulation of the morpholinyl anthracycline derivative being utilized, the particular pharmaceutical formulation of Cox-2 inhibitor being utilized, the particular cancer being treated, the severity of the disease state being treated, and the particular patient being treated.

The dosage ranges for the administration of the combined preparations according to the invention may vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art.

The dosage regimen must therefore be tailored to the particular of the patient's conditions, response and associate treatments, in a manner, which is conventional for any therapy, and may need to be adjusted in response to changes in conditions and/or in light of other clinical conditions.

When one or more active constituents of the combined preparation according to the invention are supplied along with a pharmaceutically acceptable carrier, a pharmaceutical composition is formed. A pharmaceutical composition of the present invention is directed to a composition suitable for the treatment of cancer. The pharmaceutical composition comprises a pharmaceutically acceptable carrier or excipient, and a morpholinyl anthracycline derivative of formula (I), formula (II) or a pharmaceutically acceptable salt or metabolite thereof and a cyclooxygenase-2 selective inhibitor as active ingredients.

Pharmaceutical compositions may also include stabilizers, anti-oxidants, colorants, and diluents. Pharmaceutically acceptable carriers and additives are chosen such that side effects from the pharmaceutical compound are minimized and the performance of the compound is not canceled or inhibited to such an extent that treatment is ineffective.

It is therefore a further object of the present invention a pharmaceutical composition comprising a pharmaceutically acceptable carrier or excipient and, as an active ingredient, a morpholinyl anthracycline derivative of formula (I), formula (II) or a pharmaceutically acceptable salt or metabolite thereof and a Cox-2 inhibitor.

Pharmaceutically acceptable carriers or excipients to be utilized in the preparation of a pharmaceutical composition according to the invention are well known to people skill in the art of formulating compounds in a form of pharmaceutical compositions.

For example, such pharmaceutical compositions may routinely contain, e.g., pharmaceutically acceptable salts, buffering agents, preservatives and/or compatible carriers. As used herein, "pharmaceutically acceptable carrier" refers to one or more compatible solid or liquid filler, diluent or encapsulating substances which are suitable for administration to mammals including humans.

Pharmaceutical compositions suitable for parenteral or intrahepatic administration are formulated in a sterile form.

The sterile composition thus may be a sterile solution or suspension in a non-toxic parenterally acceptable diluent or solvent.

Pharmaceutical compositions for intrahepatic administration are formulated, for example, in a form, which remains selectively in a liver tumor after their injection through the hepatic artery; LIPIODOL ™ may be a suitable carrier of anticancer agents, which can be used for intrahepatic administration.

The amount of an active ingredient contained in the pharmaceutical composition according to the invention may vary quite widely depending upon many factors such as e.g. the administration route and the vehicle.
As an example, the pharmaceutical composition of the invention may contain from 0.1 mg to 100 mg of nemorubicin; from 50 mg to 1000 mg of a Cox-2 inhibitor such as celecoxib or from 1mg to 100 mg of valdecoxib or from 5 mg to 1000 mg of rofecoxib or from 10 mg to 1000 mg of etoricoxib.

A further aspect of the present invention is to provide a method for the treatment of cancer in a subject in need of such a treatment, the method comprising administering to said subject a therapeutically effective amount of a morpholinyl anthracycline derivative of formula (I), formula (II) or a pharmaceutically acceptable salt or metabolite thereof and a Cox-2 inhibitor, in amounts effective to produce a synergistic anticancer effect.

In the method of the subject invention, a morpholinyl anthracycline derivative of formula (I), formula (II), a pharmaceutically acceptable salt or metabolite thereof may be administered simultaneously with a Cox-2 inhibitor, or the compounds may be administered sequentially, in either order. It will be appreciated that the actual preferred method and order of administration will vary according to, inter alia, the particular formulation of the morpholinyl anthracycline derivative being utilized, the particular formulation of the Cox-2 inhibitor being utilized, the particular tumor model being treated, and the particular host being treated.

In the method according to the present invention, the amount of a morpholinyl anthracycline derivative, together with the amount of the cyclooxygenase-2 selective inhibitor or prodrug thereof, constitute an amount effective for the treatment of cancer.

In the method of the subject invention, for the administration of a morpholinyl anthracycline derivative according to the invention, e.g. nemorubicin, the

course of therapy generally employed is from about 0.1 mg/m$^2$ to about 100 mg/m$^2$ of body surface area. More preferably, the course of therapy employed is from about about 1 mg/m$^2$ to about 1000 mg/m$^2$ of body surface area.

In the method of the subject invention, for the administration of a Cox-2 inhibitor, the course of therapy generally employed is within a range of from about 0.01 to about 100 mg/day per kg of body weight of the subject, preferably within a range of from about 1 to about 20 mg/day per kg of body weight of the subject.

The present invention also provides a therapeutic kit comprising, in suitable container means, a pharmaceutical formulation comprising morpholinyl anthracycline derivative of formula (I), formula (II), a pharmaceutically acceptable salt or pharmaceutically active metabolite thereof and a pharmaceutical formulation comprising a Cox-2 inhibitor are present within a single container means or within distinct container means.

As a particular example, a kit comprises a pharmaceutical formulation of nemorubicin and a pharmaceutical formulation of Cox-2 inhibitor, within distinct container means.
Kits according to the invention are intended for use in anticancer therapy as defined above.

The superadditive antitumor effect of the combination preparation of the present invention can be shown by testing the in vitro cytotoxic activity and apoptotic effect of nemorubicin and celecoxib on human tumor cell lines. Cytotoxicity is determined by microculture tetrazolium dye assay. The apoptotic effect is tested by morphology and TUNEL assays. Cox-1 and Cox-2 expression is demonstrable in all tested tumor cell lines. Cox-2 inhibition induced by celecoxib is apoptotic and reduces tumor cell proliferation. Nemorubicin is highly cytotoxic against all tested cell lines. The combination

of nemorubicin and celecoxib is supraadditive/synergistic, as assessed by isobolographic analysis. Supra-additive/synergistic is observed irrespective of treatment sequence.